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TITLE: Development of Triplex-Forming Oligonucleotides to Inhibit Expression of the c-myc Oncogene in Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Eileen M. McGuffie, Ph.D.
Carlo V. Catapano, M.D.

CONTRACTING ORGANIZATION: Medical University of South Carolina
Charleston, South Carolina 29425

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6. AUTHOR(S)
Eileen M. McGuffie, Ph.D.
Carlo V. Catapano, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Medical University of South Carolina
Charleston, South Carolina 29425

E-Mail: mcguffem@musc.edu

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13. ABSTRACT ***(Maximum 200 Words)***

Triple helix-forming oligonucleotides (TFOs) bind with high affinity and specificity to homopurine/homopyrimidine sequences in double-stranded DNA. Oligonucleotide-directed triplex formation in gene promoter regions has been shown to inhibit transcription of targeted genes. *C-myc* is a growth-promoting oncogene that is deregulated and overexpressed in breast and other cancers. Our research is designed to assess TFO-mediated reduction of c-myc expression as a means of decreasing breast tumor growth. The purpose of the present work was to optimize the binding affinity and nuclease resistance of a previously studied myc-targeted TFO. A novel chimeric TFO was designed, consisting of two portions with dissimilar nucleotide content and opposite polarity. This TFO bound with approximately tenfold higher affinity than the TFO used in our previous study, and its double 5' ends conferred increased resistance to degradation by nucleases *in vitro*. Studies of the chimeric TFO's anti-gene activity *in vitro* and in cells are in progress. Further modifications may be added to enhance nuclease resistance in cells. In another series of experiments, we found that conjugation of a short experimental TFO to daunomycin, an intercalating agent, resulted in more stable triplex without loss of binding specificity. Further testing of daunomycin-conjugated TFOs is planned.

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Description of training and research accomplishments.

The approved proposal described three tasks. Progress made on each task during the first year is outlined below. Task 1 was planned to take up most of this year and is discussed in detail. Preliminary work on tasks 2 and 3 is briefly described.

Task 1. To design and evaluate activity of *c-myc* TFOs optimized for nuclease resistance and high-affinity DNA binding (months 1-10).

- Design and oversee synthesis of modified TFOs. Compare affinity and specificity of in vitro binding of modified and PS-TFOs and assess nuclease resistance. Identify modified TFOs with binding affinity superior to PS-TFOs, but with similar specificity and nuclease resistance
- Determine whether binding properties and nuclease resistance of TFOs are further improved by attachment of an intercalating agent to either the 5' or 3' terminus.

Work done during the first year of the training award has focussed on Task 1. We have concentrated on improving the binding affinity and stability of a triple helix-forming oligonucleotide (TFO) directed to a sequence in the P2 promoter region of the c-myc gene. An improved TFO has been designed, and studies have been initiated to examine the effects of attaching an intercalating agent to the TFO.

The research described in the approved proposal builds on previous studies conducted in the mentor's laboratory and elsewhere. A variety of TFOs has been shown to bind with high affinity and specificity to homopurine-homopyrimidine sequences in double-stranded DNA. TFOs targeted to regulatory sequences in target genes have been able to selectively reduce gene transcription. This approach has potential application in anti-cancer treatment since it can be used to reduce levels of proteins essential to proliferation of cancer cells. C-Myc plays a central role in promoting cell growth and proliferation. The *c-myc* gene is expressed at abnormally high levels in many cancers, and its overexpression has been strongly associated with initiation and progression of breast cancer. Reduced levels of c-Myc are associated with decreased breast cancer cell growth. The purpose of our investigation is to assess triplex DNA-mediated reduction of *c-myc* expression as a potential means of decreasing growth and spread of breast tumors.

Several sequences suitable for triplex formation are present in the *c-myc* gene. In a study comparing TFOs directed to a subset of these sequences, we found that a TFO targeted to a critical regulatory region close to the P2 promoter had strong anti-gene and anti-proliferative effects in leukemia and lymphoma cells. These findings encouraged further development of this TFO. The specific problems addressed by the present research arose from modifications required to confer nuclease resistance on the c-myc TFO. Nuclease resistance is required in TFOs intended for *in vivo* use. Replacement of nuclease-sensitive phosphodiester (PO) internucleotide linkages with resistant phosphorothioate (PS) appeared to reduce the binding affinity of the c-myc TFO. Furthermore, adverse effects of high concentrations of PS-oligonucleotides have been observed in cells and animals. We therefore decided to investigate modifications in design, which could enhance TFO binding affinity, and retain nuclease resistance while allowing reduced PS content.

Our initial studies of binding affinity focussed on the nucleotide composition of the Myc P2-targeted TFO. It has been established that nucleotide composition determines the binding characteristics of TFOs. Thus, oligonucleotides composed of pyrimidine bases (C and T) bind in parallel orientation to the homopurine strand of the target duplex, forming C^+.G-C and T.A-T triplets. TFOs composed of purine bases (G and A), or of mixed purine/pyrimidine (G and T) bind in anti-parallel orientation to the homopurine strand, forming G.G-C and A.A-T or T.A-T triplets. In certain circumstances GT- TFOs can bind in parallel orientation. A parallel-binding GTC-TFO has also been characterized. Stability of C^+.G-C triplets depends on protonation of cytosines, so that binding of CT-TFOs in the parallel motif is favored by acidic pH. Triplex formation by GA and GT TFOs is pH independent. Binding in each of these motifs has been shown to occur under physiological conditions, although cytosines modified by methylation are required to overcome

the pH dependence of CT TFOs. As a general rule, triplex formation in the anti-parallel motif is favored when the purine strand of the target duplex is G-rich, while A-rich target sequences are more favorable to parallel binding.

The myc P2 target purine strand is almost equally comprised of A and G, although the distribution is uneven, with A grouped at the 5', and G at the 3' end. We previously found that an antiparallel motif phosphodiester GT-TFO bound the P2 sequence with reasonably high affinity, although affinity decreased significantly upon phosphorothioate modification of the TFO. We investigated whether an alternative binding motif might be favorable, given the peculiarities of the target sequence. Using electrophoretic mobility shift assays (EMSA), we measured binding affinities of a series of short 11-mer PO-TFOs targeted to either the A-rich 5' or the G-rich 3' segment of the 23 base pair target duplex. We tested GT-TFOs in the parallel and anti-parallel motifs; GA-TFOs in the anti-parallel motif, and CT- and GTC-TFOs in the parallel motif. This approach allowed us to identify the preferred binding motif for each segment of the target duplex. In comparing TFOs targeted to the G-rich 3' sequence, we determined that an 11 mer GT-TFO designed to bind in the antiparallel motif bound with highest affinity. These 11 bases form part of the 23-mer TFO previously found to have antigene activity. Although an anti-parallel GA-TFO was also able to form triplex, efficiency was very low due to its apparent propensity to form self-aggregates. No other short TFO was able to form stable triplex with the G-rich region. We then tested a series of TFOs targeted to the A-rich segment of the target. Interestingly, only a CT-TFO, binding in parallel orientation and at acidic pH, was able to form a stable triplex.

These results suggested that the preferred TFO binding motif varied within the 23 base pair Myc P2 target sequence. The G-rich 3' segment was most efficiently bound by an anti-parallel GT-TFO, while the A-rich 5' segment required a parallel CT-TFO for stable triplex formation. Short TFOs were not candidates for further development as *in vivo* anti-gene agents however, since a length of at least 15 bases is required to ensure target specificity. Furthermore, stability of triple helix is reduced as the number of base triplets decreases. Therefore, to optimize TFO binding affinity while retaining specificity and stability, we designed a novel chimeric GTC-TFO, consisting of the GT- and CT-TFOs synthesized to be contiguous but with opposite polarity. This design resulted in a TFO with two 5' ends and a central 3'-3' linkage. Cytosines were methylated at the C5 position to allow binding of the CT portion at physiological pH. We then compared triplex formation by the chimeric TFO and the 23 mer GT-TFO. EMSA and dimethyl sulfate protection assays showed that the GTC-TFO formed triple helix with equally high specificity and significantly improved affinity and stability compared to the anti-parallel GT-TFO. We did not include the GT-TFO with PS linkages in these studies since it was already known to have lower binding affinity than the PO-TFO. A control chimeric oligonucleotide with identical but scrambled nucleotide content did not form triple helix. To the best of our knowledge, this is the first chimeric TFO to be designed and used in this way.

Since the improved binding properties of the GTC-TFO encouraged further development leading to testing *in vivo*, we next addressed the question of nuclease resistance. To determine if the double 5'-end structure would confer resistance to 3'-exonuclease activity, we compared *in vitro* sensitivity of the GTC- and the GT-TFOs to nucleases present in fetal bovine serum. We found that the GTC-TFO was stable for at least 24 h while the GT-TFO was significantly degraded. We then tested resistance to intracellular nucleases by transfecting MDA-MB-231 breast cancer cells with 5'-radiolabeled GTC-TFO. In this environment, degradation of the TFO was observed within 6 h and by 48 h little intact TFO remained. These results suggested that enhanced nuclease resistance due to lack of a 3' end, was insufficient to ensure long-term survival in cells. Several possible strategies to protect the TFO in the cellular environment are currently under consideration. The CT portion of the TFO can be synthesized as RNA with alkylated 2' hydroxyl groups (2'0-Me RNA). This modification has been shown to improve binding affinity as well as conferring nuclease resistance. One to three PS linkages can be added at the double 5'ends, or alternatively, the GT portion can be entirely composed of phosphorothioates. Curiously, full PS modification has been found by others to severely reduce binding by parallel motif TFOs, while 2'O-Me RNA has not been found to support triplex formation in the anti-parallel orientation.

These limitations will help guide the choice of strategy. So far, we have not directly compared the activity of the GTC-TFO to that of a PS-TFO. These studies will be done when a fully nuclease resistant chimeric TFO has been synthesized.

Attachment of an intercalating agent to the 5' or 3' end of a TFO has been shown to stabilize triple helix. This modification may also improve nuclease resistance. We therefore initiated studies to determine the effects of conjugating myc P2 TFOs to daunomycin, an anti-tumor drug and potent DNA intercalator. This work was done in collaboration with Dr. Massimo L. Capobianco, I.Co.CEA-CNR, Bologna Italy. Dr. Capobianco was the first to synthesize daunomycin-conjugated oligonucleotides. Daunomycin-TFOs (D-TFOs) tested so far include the short GT-TFO found to bind with high affinity to the G-rich sequence of the P2 target. As shown by EMSA, this D-TFO formed a much more stable triplex than the non-conjugated TFO. Furthermore, DMS footprinting showed that the D-TFO protected only the short 11bp target sequence, suggesting that the intercalating agent did not affect binding specificity. We also found that this D-TFO inhibited *in vitro* and *in vivo* transcription of *c-myc.* These results are described in more detail below. Although short TFOs are not stable or specific enough for use *in vivo,* as discussed above, their weaknesses constitute a stringent test of the effects of an intercalator on stability and specificity. Further studies are planned using short D-TFOs, and this modification is likely to be added to the GTC-TFO and other full-length TFOs.

Task 2. To enhance efficiency of cell internalization, maximize stability, and compare growth-inhibitory activities of PS- and modified TFOs in estrogen-dependent and –independent breast cancer cells (months 10-18).
Task 3. To extensively characterize anti-gene activity, growth inhibitory activity, and triplex forming ability of TFOs in breast cancer cells (months 18-36).

In preparation for accomplishing Tasks 2 and 3, which focus on extensively characterizing anti-gene and intracellular activity of optimized c-myc TFOs, we have developed several reporter systems that will facilitate measurement of TFO-mediated inhibition of gene transcription. These systems provide rigorous tests of a TFO's ability to compete with regulatory proteins for DNA-binding *in vitro* and in cells. TFOs that perform efficiently in reporter assays are clearly suitable for further testing and structural modification. This approach was chosen as the most cost-effective way to screen candidate TFOs before adding further structural modifications for long-term *in vivo* stability. For *in vitro* tests, nuclear extracts have been prepared from MCF-7 and MDA-MB-231 breast cancer cells. These are being used in gel shift assays to measure the ability of TFOs to compete with nuclear proteins for binding to a labeled probe containing the myc P2 target sequence. Nuclear extracts are also used for *in vitro* transcription assays. Here, plasmid DNA containing the *c-myc* promoter region provides the template for transcriptional activity present in the extracts. TFOs will be tested for their ability to inhibit transcription, as measured by electrophoretic examination of radiolabeled reaction products. An assay system for use in cells has also been designed. Plasmids have been constructed in which sequences containing various portions of the *c-myc* regulatory region control transcription of a luciferase reporter gene. Plasmids are introduced into breast cancer cells by transfection, along with TFOs or control oligos, then luciferase activity is measured at successive intervals. This approach allows sensitive and accurate quantitation of the effects of TFOs on target gene transcription. Preliminary results using reporter systems have been obtained with the D-TFO described above. The D-TFO showed inhibitory effects on transcription *in vitro,* and caused an approximate 50% reduction in expression of the luciferase gene in MCF-7 and MDA-MB-231 cells. A control D-TFO had no such effects. Studies are continuing with this TFO and are being initiated with the chimeric GTC-TFO.

Key accomplishments.

- A *c-myc*-targeted TFO with greatly improved binding affinity has been designed and tested *in vitro*
- TFOs conjugated to daunomycin, a DNA intercalating agent have been synthesized and tested *in vitro* and in breast cancer cells
- C-myc promoter-driven reporter plasmids have been constructed and tested for activity in breast cancer cells
- Reporter assays have been developed for efficient screening of candidate c-myc TFOs.

Reportable outcomes

Publications

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